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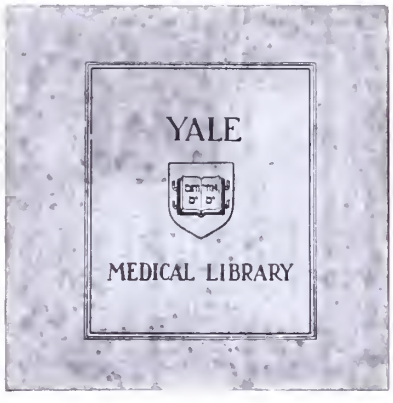
THE INFLUENCES OF CYTOTOXIC DRUGS AND L1210 SUSPENSION CULTURE DENSITY ON DE NOVO PURINE SYNTHESIS

Joyce Ann O'Shaughnessy

1982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The Influences of Cytotoxic Drugs and L1210 Suspension Culture Density on de novo Purine Synthesis

A thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the degree of Doctor of Medicine to be awarded in May, 1982.

Joyce Ann O'Shaughnessy

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Acknowledgements:

I wish to thank Ed Cadman and Chris Benz for four years of guidance and support and for the opportunity to work with them, and Robert Heimer for his assistance and advice. I am grateful for the funding provided by Ed Cadman and the Yale School of Medicine Summer Student Fellowship Program which supported this work.

ABSTRACT

Pretreatment of L1210 cells with methotrexate has been shown by E. Cadman et al to enhance intracellular accumulation of 5-fluorouracil and the formation of metabolically active fluoropyrimidines. Sequential treatment with methotrexate and 5-fluorouracil resulted in synergistic cytotoxicity. These effects of methotrexate were correlated with increased intracellular levels of PRPP(5-phosphoribosyl-1-pyrophosphate), the cofactor required for the conversion of 5-fluorouracil to 5-fluorouridine-5'-monophosphate(FUMP). Methotrexate is a potent inhibitor of de novo purine synthesis and it is likely that methotrexate's anti-purine effect resulted in the increased PRPP levels and therefore in synergism with 5-fluorouracil. The anti-purine effects of 24 cytotoxic agents were measured by quantitating incorporation of 1-^{14}C-glycine into purine bases. Possible synergism between each of these agents and 5-fluorouracil is discussed based on the agents' mechanisms of de novo purine synthesis inhibition.

It was found that de novo purine synthesis progressively decreased from peak values in early log phase growth to $\sim$ 15% of the maximal level during late log and plateau phase growth of L1210 cultures. Increasing concentrations of exogenous hypoxanthine, a substrate in the salvage pathway of purine biosynthesis, were shown to progressively decrease de novo purine synthesis, making increased utilization of salvage purine synthesis along the growth curve one possible explanation for the observed decline in de novo purine synthesis.

A reduced rate of de novo purine synthesis secondary to lengthening of the cell cycle from early to late log phase growth is discussed as a second explanation. Finally, a progressive decrease in cell volume with increasing culture density resulting in a need for reduced overall purine synthesis to maintain the required intracellular purine nucleotide concentrations is discussed as a third possible explanation for the decrease in de novo purine synthesis. The anti-purine effects of cytotoxic agents must be interpreted in the light of this natural progressive decline in de novo purine synthesis during exponential cell growth.

INTRODUCTION

In treating cancer with cytotoxic drugs, clinical trials have shown that combinations of drugs generally result in better tumor response and patient survival than does single agent chemotherapy. Until recently these drug combinations were most often chosen on the basis of different proposed sites of drug action, different toxicity or simply empirically. Rarely has sequential drug therapy been designed from known biochemical parameters that would facilitate effective drug activation or other advantageous interactions between drugs or between natural metabolites and drugs. Certain sequential drug combinations have demonstrated synergism in inhibiting the growth of animal tumors(1), and the biochemically rational design of sequential drug therapy has become of interest to many investigators.

Methotrexate and 5-fluorouracil have been used successfully both singly and in combination in treating many human neoplasms including breast and colon cancer(2). Ed Cadman et al have shown that methotrexate pretreatment of L1210 murine leukemia cells in concentrations which produced near maximal inhibition of dihydrofolate reductase, enhanced intracellular accumulation of 5-fluorouracil(5-fold) and increased 5-fold the formation of the 5-fluorouracil nucleotides, FUMP, FUDP, FUTP and FdUMP. These alterations correlated with synergistic cytotoxicity. In cells pretreated with methotrexate in concentrations between 0.1μM and 10μM, intracellular 5-phosphoribosyl-1-pyrophosphate(PRPP) pools were increased between 2 to 8 times over control values. The increases in

PRPP levels and in 5-fluorouracil accumulation could be prevented by the addition of leukovorin(N^5-formyltetrahydrofolate) in concentrations which rescued cells from the inhibitory effects of methotrexate. If the PRPP pools were reduced following methotrexate pretreatment by adding hypoxanthine which uses PRPP in its conversion to inosone monophosphate(IMP), the intracellular accumulation of 5-fluorouracil was not enhanced. These observations indicate that the enhanced 5-fluorouracil accumulation within L1210 cells following methotrexate pretreatment resulted from methotrexate's anti-purine effect which led to increased PRPP levels. The augmented PRPP levels produced by methotrexate were most likely the result of decreased PRPP utilization in de novo purine synthesis and decreased endproduct inhibition of PRPP synthetase by the reduction in the purine nucleotides. The augmented PRPP pools were then able to be utilized in the conversion of 5-fluorouracil to 5-fluorouridylate(5FUMP) by orotate phosphoribosyltransferase, the rate-limiting step in 5-fluorouracil intracellular metabolism(4). This is the primary reaction regulating the rate of intracellular 5-fluorouracil accumulation. Methotrexate pretreatment of L1210 cells then incubated with 5-fluorouracil resulted in a 5-fold increased incorporation of 5-fluorouracil into RNA and in synergistic cell death as determined by soft agar cloning. Methotrexate pretreatment not only inhibits de novo purine synthesis leading to increased PRPP levels, but it also inhibits thymidylate(dTMP) synthesis. 5-fluorouracil, in one of its metabolically active forms, 5-fluoro-2'-deoxyuridine-5'-monophosphate(5-FdUMP),

also inhibits thymidylate synthesis by inhibiting the enzyme, thymidylate synthetase. Since the increased formation of 5-FdUMP is unlikely to substantially increase the inhibition of thymidylate synthesis induced by methotrexate pretreatment, the likely mechanism of methotrexate - 5-fluorouracil synergistic cytotoxicity is the enhanced incorporation of the metabolite fluorouridine triphosphate(FUTP) into RNA(3). This proposed mechanism of methotrexate - 5-fluorouracil synergism was tested in monolayer cultures of human colorectal adenocarcinoma, HCT-8 cells. Sequential treatment with methotrexate and 5-fluorouracil resulted in a 3-fold increase in total intracellular 5-fluorouracil accumulation, increased PRPP pools associated with enhanced 5-fluorouracil metabolism, and synergistic inhibition of human colorectal tumor cells' clonal growth(5,6).

The hypothesis of Cadman et al is that methotrexate enhances the metabolism of 5-fluorouracil to fluoronucleotides which are incorporated into RNA, by inhibiting de novo purine synthesis. Other inhibitors of de novo purine synthesis, therefore, might prove to be similarly synergistic with 5-fluorouracil. The purpose of this work was to quantitate the degree of de novo purine synthesis inhibition by several purine and pyrimidine analogs and antagonists, nucleosides and other cytotoxic agents by measuring the amount of 1-^{14}C-glycine incorporated into adenine and guanine. Additional work performed by Cadman et al investigated 5-fluorouracil intracellular accumulation, PRPP pools, fluoronucleotide levels and cytotoxicity resulting from sequential treatment

of L1210 cultures with several anti-purine agents and 5-fluorouracil. The combined investigations were performed to determine whether cytotoxic synergism of 5-fluorouracil and another anti-tumor agent was related to the agent's ability to inhibit de novo purine synthesis. In addition, this study investigates the relationship between the growth density of L1210 suspension cultures and the rate of de novo purine synthesis, examining the purine salvage pathway as a possible alternative method of purine synthesis at high culture densities. Cell cycle length and cell volume are also discussed as factors influencing the rate of de novo purine synthesis.

MATERIALS AND METHODS

Cells

L1210 murine leukemia cells with a doubling time of 10 to 12 hours were maintained as stationary suspension cultures in Fischer's glycine-free medium plus 10% horse serum and were grown at 37°C in a 5% CO_2 atmosphere. The stocks were diluted twice weekly and monthly cultures for mycoplasma contamination were negative. For each experiment testing a proposed anti-purine agent, cells were inoculated at 1-2 X 10^4cells/cc and were harvested in the logarithmic phase of growth, generally at 2-5 X 10^5cells/cc. Staining with trypan blue demonstrated $\sim$ 99% viability until cultures entered plateau phase. After 120 hours in culture, viability was reduced to $\leq$ 70%(7). Cells were counted with a model ZBI Coulter Counter(Coulter Electronics, Inc., Hialeah, FL).

Drugs

1-^{14}C-glycine(20mCi/mmole) which was used to evaluate de novo purine synthesis was obtained from the New England Nuclear Corporation(Boston, MA). 8-^{14}C-hypoxanthine (56mCi/mmole) which was used in assessing the purine salvage pathway of L1210 cells was also obtained from the New England Nuclear Corporation(Boston,MA). Methotrexate, N-(phosphonacetyl)-L-aspartate(PALA), vincristine, 5-azacytidine and 3-deazauridine were provided by the Drug Development Branch of the National Cancer Institute, Bethesda, MD. Pyrazofurin was provided by Lilly Co.(Indianapolis,IN). All other compounds were purchased from Sigma(St. Louis,MO).

Growth Curve

Logarithmically growing L1210 cells were inoculated into Fischer's medium plus 10% horse serum to achieve a concentration of 1 X 10^4cells/cc. Aliquots of 10cc were transferred to sterile culture tubes(3 tubes/time point). At 24-hour intervals, the tubes were carefully agitated and 1-cc aliquots were each diluted with 9cc of 0.9% NaCl. The cells were dispersed with gentle pipetting and cell numbers were determined with the ZBI Coulter Counter.

De Novo Purine Synthesis Determination

The incorporation of 1-^{14}C-glycine into the purine bases of nucleic acid was used as a measure of de novo purine synthesis. By a modification of Henderson's technique (8), 100cc aliquots of L1210 cells at various phases of suspension growth were incubated with 5μM 1-^{14}C-glycine for 2 hours. In some experiments, cell cultures of 2-5 X 10^5cells per cc were incubated with anti-tumor agents for 1-2 hours before the 1-^{14}C-glycine was added. The cells were centrifuged at 1000 X g for 5 mins. and were washed in cold phosphate buffered solution(PBS) with 1mM glycine(unlabelled) three times. The supernatant was discarded and the cell pellet was incubated with 1.5cc of 1M$HClO_4$ at 100°C for 1 hour to depurinate the nucleic acids. Following centrifugation at 1000 X g for 10 mins., the acid soluble supernatant was treated with 4N KOH to precipitate out the perchlorate salt. The supernatant, containing free purine bases, was neutralized with HCl and was analyzed by high pressure liquid

chromatography(HPLC) on a Partisil ODS-2 column(Whatman, Inc., Clifton, NJ) eluting with a 0.1M sodium acetate buffer(pH 5.5) and acetonitrile as a linear gradient from 0 to 7.5% over 30 mins. Nonradiolabelled adenine and guanine were used as markers to determime column retention times: Void volume containing glycine that was not incorporated into purine bases, $\simeq$ 4-7 mins., Guanine $\simeq$ 12 mins. and Adenine $\simeq$ 38 mins. Absorbance was recorded at 254 and 280nm, 0.5ml fractions were collected and the radioactivity was quantitated. The amount of radiolabel incorporated into adenine and guanine was calculated as pmol/10^6cells/hour, was expressed as a percentage of control and reflected the de novo purine synthesis rate for each experimental condition. The control cultures for each study were harvested for testing at the same culture density and with the same nutritional status as the experimental cultures.

RESULTS

Effects of Anti-tumor Agents on de novo Purine Synthesis

The effects of 24 anti-tumor agents on the incorporation of 1-^{14}C-glycine into purine bases are shown in Table 1 and in Figure A. In general, the purine analogs and antagonists such as hypoxanthine, inosine, adenosine, tubercidin and 6-thioguanine were found to be more potent inhibitors of de novo purine synthesis than were the pyrimidine analogs and antagonists such as 3-deazauridine, pyrazofurin, PALA, Ara-C, thymidine and 5-azacytidine. The RNA and protein synthesis inhibitors, actinomycin and cycloheximide respectively, were also found to significantly reduce de novo purine synthesis.

Effects of Culture Density on de novo Purine Synthesis

It was observed that the ability of an anti-tumor agent to inhibit de novo purine synthesis was dependent on the density of the culture that the experimental cells were harvested from. Table 2 illustrates that, in the case of 5-fluorouracil, the anti-tumor agent could inhibit de novo purine synthesis to a nearly identical percentage of control given the same experimental culture concentration. Seen in the case of methotrexate, however, is an inverse relationship between culture density and the degree to which this potent anti-purine agent can inhibit de novo purine synthesis. The control value for the amount of 1-^{14}C-glycine incorporated into adenine and guanine in the methotrexate study performed at a density of 1.2 X 10^5cells/cc was 25.5pmoles of

TABLE 1 1-^{14}C-glycine Incorporation into Purine Bases

Drug	Exposure Time (hrs.)	Concentration (μM)	1-^{14}C-glycine into Adenine and Guanine/10^6 cells/hr (% of control ± 2.1%)
Purine Analogs			
Hypoxanthine	3	10	13.0
Inosine	3	10	9.4
Adenosine	3	10	20.7
6-mercaptopurine	3	5	58.7
6-thioguanine	3	5	15.9
Virazole	3	10	39.1
Tubercidin	1.5	10	17.4
MMPR	3	2	61.0
Pyrimidine Analogs			
Ara-C	3	1	84.8
Thymidine	3	100	72.5
Pyrazofurin	3	5	93.8
5-azacytidine	3	30	62.6
5-fluorouracil	3	100	20.7
5-fluorouridine	3	100	26.1
5-fluorodeoxyuridine	3	100	53.3
Others			
Hydroxyurea	3	100	90.0
Methotrexate	3	10	11.3
Leukovorin	3	1000	108.2
Vincristine	3	10	15.2
Actinomycin D	3	10 g/ml	8.7
Cycloheximide	3	10	10.9
Azaserine	3	10	14.1
L-alanosine	3	2	79.0
PALA	3	1	84.8

Table 1 (continued)

Abbreviations: MMPR, 6-methyl-mercaptopurine ribonucleoside; Ara-C, 1-β-D-arabinofuranosylcytosine; PALA, N-phosphonacetyl-L-aspartate.

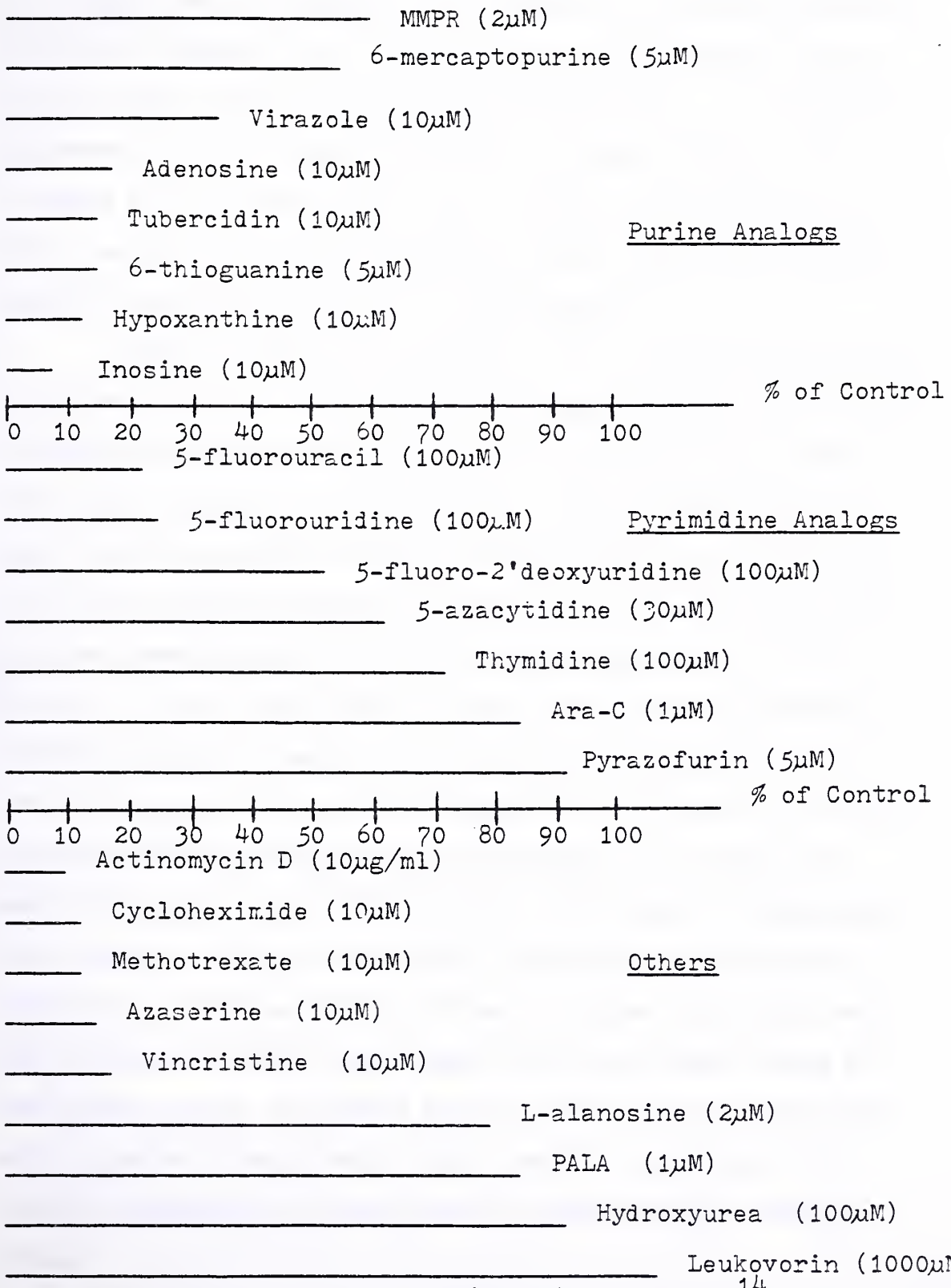
The control value of moles of 1-^{14}C-glycine incorporated into adenine and guanine/10^6cells/hr varied with the culture density and position of the control cells on the growth curve. For middle log phase cells at 3 X 10^5cells/cc, the control value was 9.76 $\pm$ 2.45 pmoles 1-^{14}C-glycine incorporated into adenine and guanine/10^6cells/hr. The "% of control" values for the anti-tumor agents represent the amount of de novo purine synthesis occurring in the presence of the anti-tumor agents relative to the control cultures. These "% of control" values were reproducible within $\pm$ 2.1% when the agents were tested in duplicate.

Table 2

Drug	Concentration (μM)	Cell Culture Concentration (X10^5cells/cc)	1-^{14}C-glycine into Adenine and Guanine % of Control $\pm$ 2.1%
5-fluorouracil	100	1.2	15.9
5-fluorouracil	100	1.3	20.7
Methotrexate	10	1.2	16.5
Methotrexate	10	6.0	50.0

As in Table 1, the control value for the amount of 1-^{14}C-glycine incorporated into purine bases varied with the culture density. For mid-log cells(3 X 10^5cells/cc) the control value was 9.76 $\pm$ 2.45pmoles 1-^{14}C-glycine into adenine and guanine/10^6cells/hr.

Figure A Inhibition of de novo Purine Synthesis by Cytotoxic Agents



Mean (100%) Control Rate = 9.76 ± 2.45 pmoles 1-^{14}C-glycine into Adenine + Guanine/10^6 cells/hr.

1-^{14}C-glycine/10^6cells/hr. The control for the methotrexate study at a density of 6 X 10^5cells/cc was 3.4pmoles incorporated into adenine and guanine/10^6cells/hr. It therefore appeared that the amount of de novo purine synthesis decreased as the density of L1210 cultures increased and that this decline in de novo purine synthesis influenced the apparent anti-purine potency of methotrexate.

Figure B depicts the growth curve of L1210 cells over a 120 hour period. The cells grow exponentially to a culture concentration of approximately 5.5 X 10^5cells/cc at which the plateau phase of growth begins. Table 3 and Figure C show the relationship between culture density and the rate of de novo purine synthesis defined as the amount of 1-^{14}C-glycine incorporated into adenine and guanine in a 2 hour period. It was found that the rate of de novo purine synthesis decreases steadily as the culture density of L1210 cells increases. As seen in Figure C, the amount of de novo purine synthesis progressively decreased to $\sim$ 15% of the early log phase level(100%), even in the face of exponential cell growth. During logarithmic cell growth, DNA and RNA synthesis procede actively. Since the rate of de novo purine synthesis clearly diminishes along the upward swing of the growth curve, it seemed possible that the salvage pathway of purine biosynthesis might provide a portion of the purine nucleotide pool required for the actively dividing cells.

To examine whether salvage purine synthesis could account for the decline in de novo purine synthesis along the growth curve, increasing concentrations of exogenous

Figure B Growth Curve of L1210 Cell Cultures

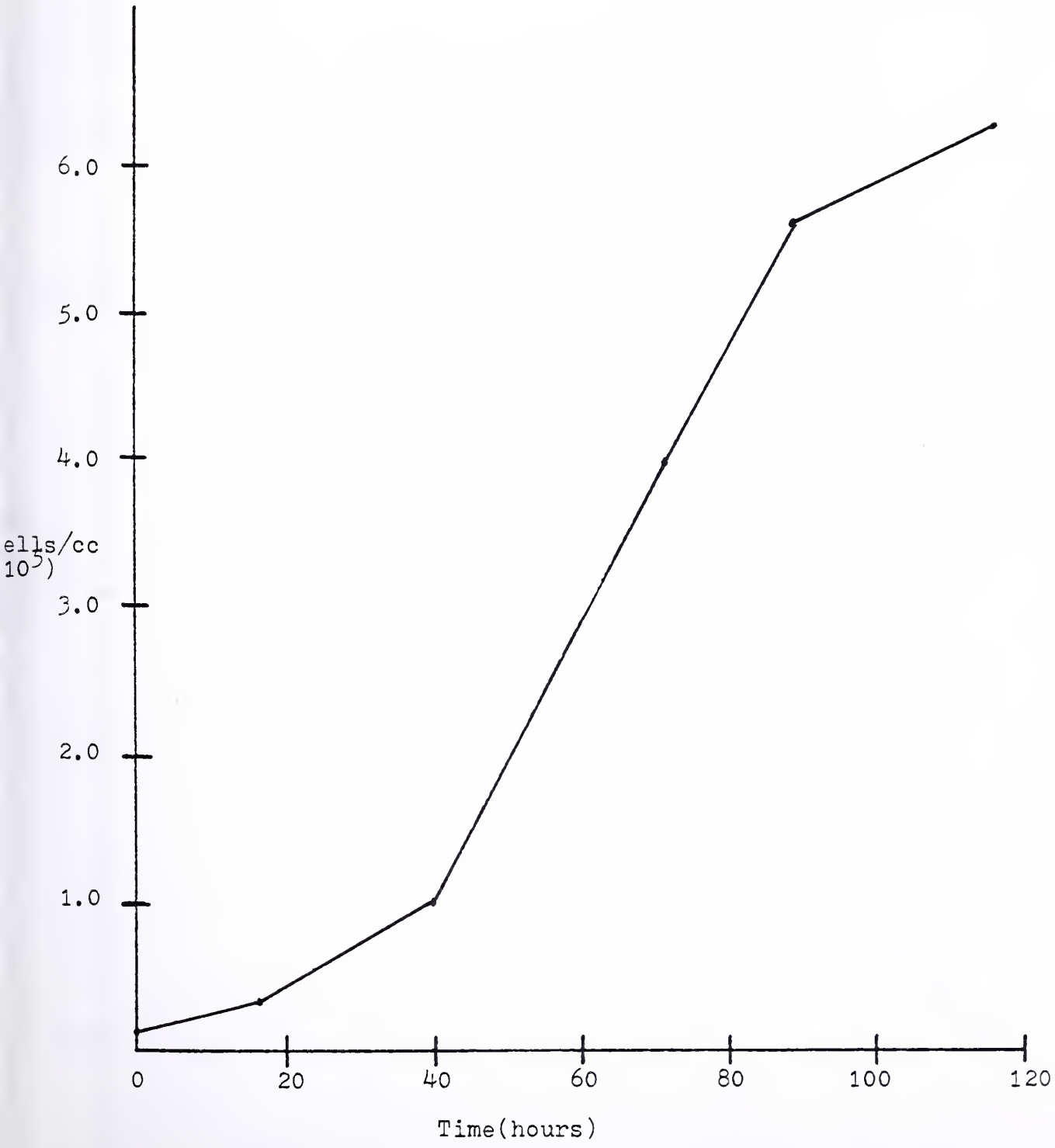


Figure C L1210 Culture Concentration vs. Rate of de novo Purine Synthesis

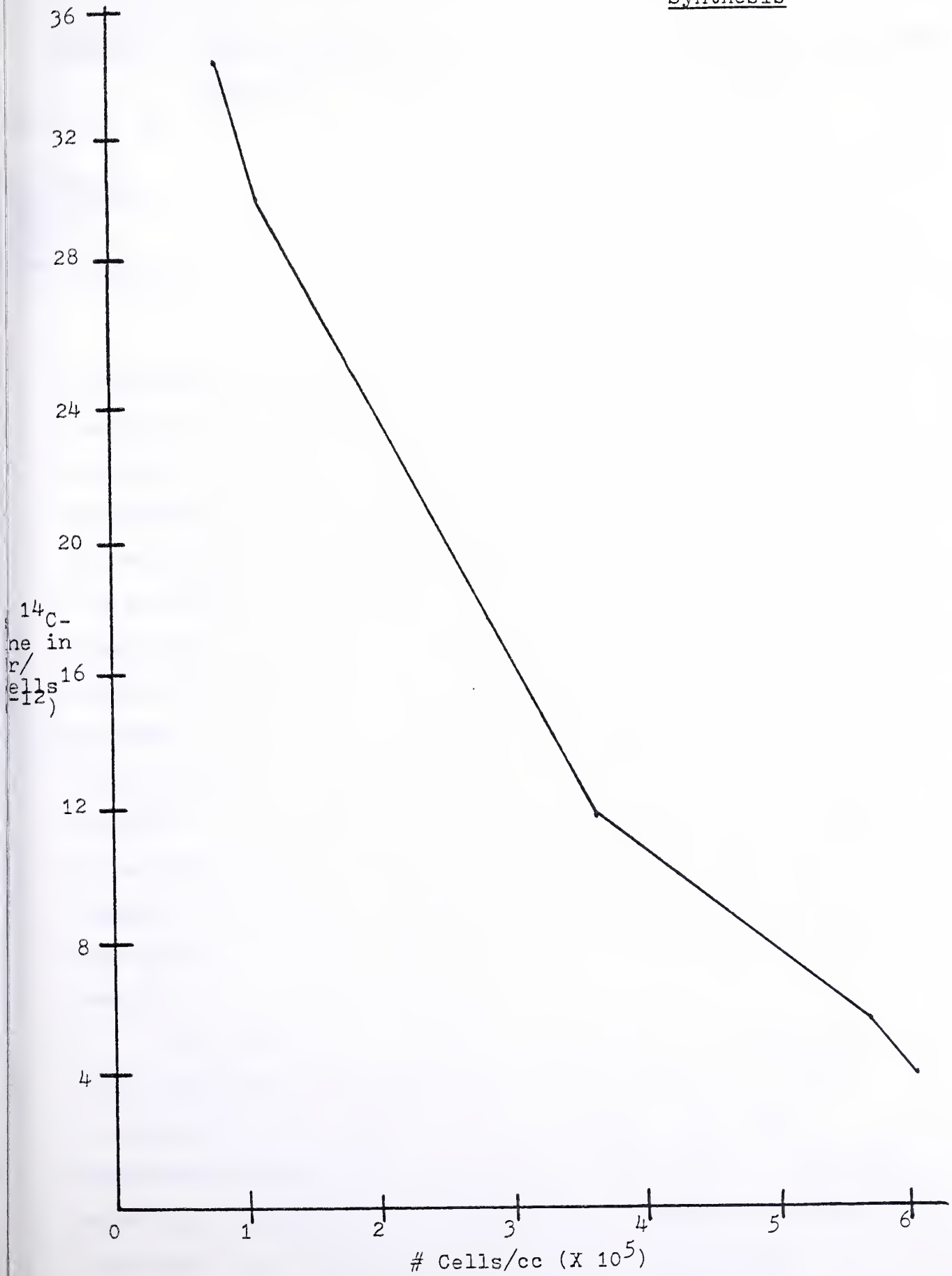


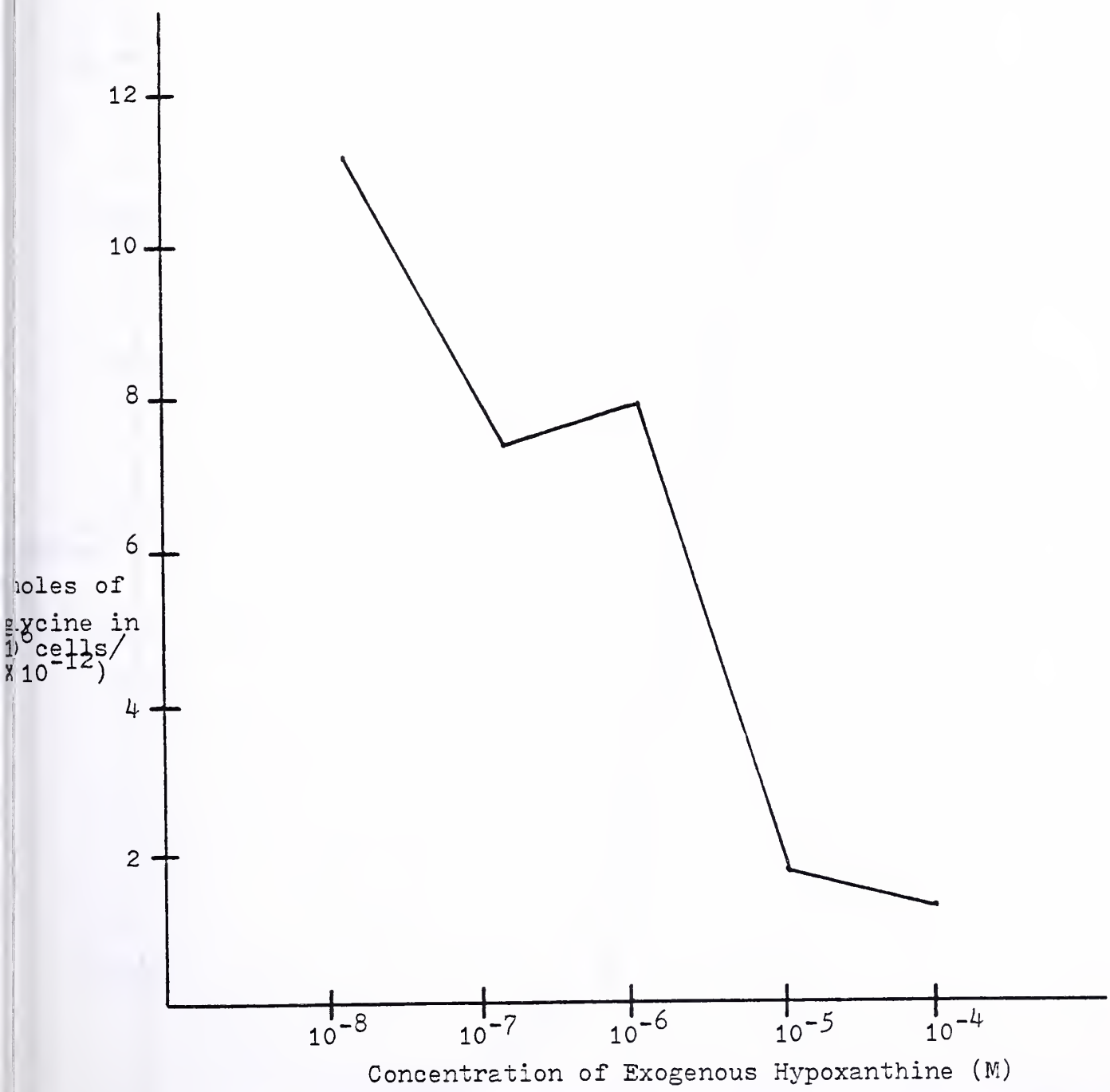
Table 3 L1210 Culture Density vs. Rate of de novo Purine Synthesis

:ells/cc X 10^5	Early Log (0.3 - 1.2)	Mid Log (1.3 - 3.5)	Late Log (3.6 - 5.6)	Plateau ($\geq$ 5.7)
ın Rate of novo Purine nthesis moles ^{14}C- ycine into /hour/10^6 cells)	31.0	20.4	9.8	5.6

hypoxanthine were incubated with L1210 cell cultures. Hypoxanthine is an intermediate of purine catabolism and can be utilized in salvage purine synthesis. Figure D shows that as the availability of hypoxanthine in the culture media increased, de novo purine synthesis was reduced to, at 10^{-5}M hypoxanthine, 17% of control. The control value for de novo purine synthesis was 10.2 pmoles 1-^{14}C-glycine incorporated into adenine and guanine/ 10^6cells/hr without any exogenous hypoxanthine in the media. The experiment was performed with L1210 cultures in log phase growth at a density of 4.0 X 10^5 cells/cc. This relationship between the progressive decrease in de novo purine synthesis and increasing concentrations of exogenous hypoxanthine suggests that utilization of the salvage pathway of purine synthesis leads to reduced purine synthesis via the de novo pathway.

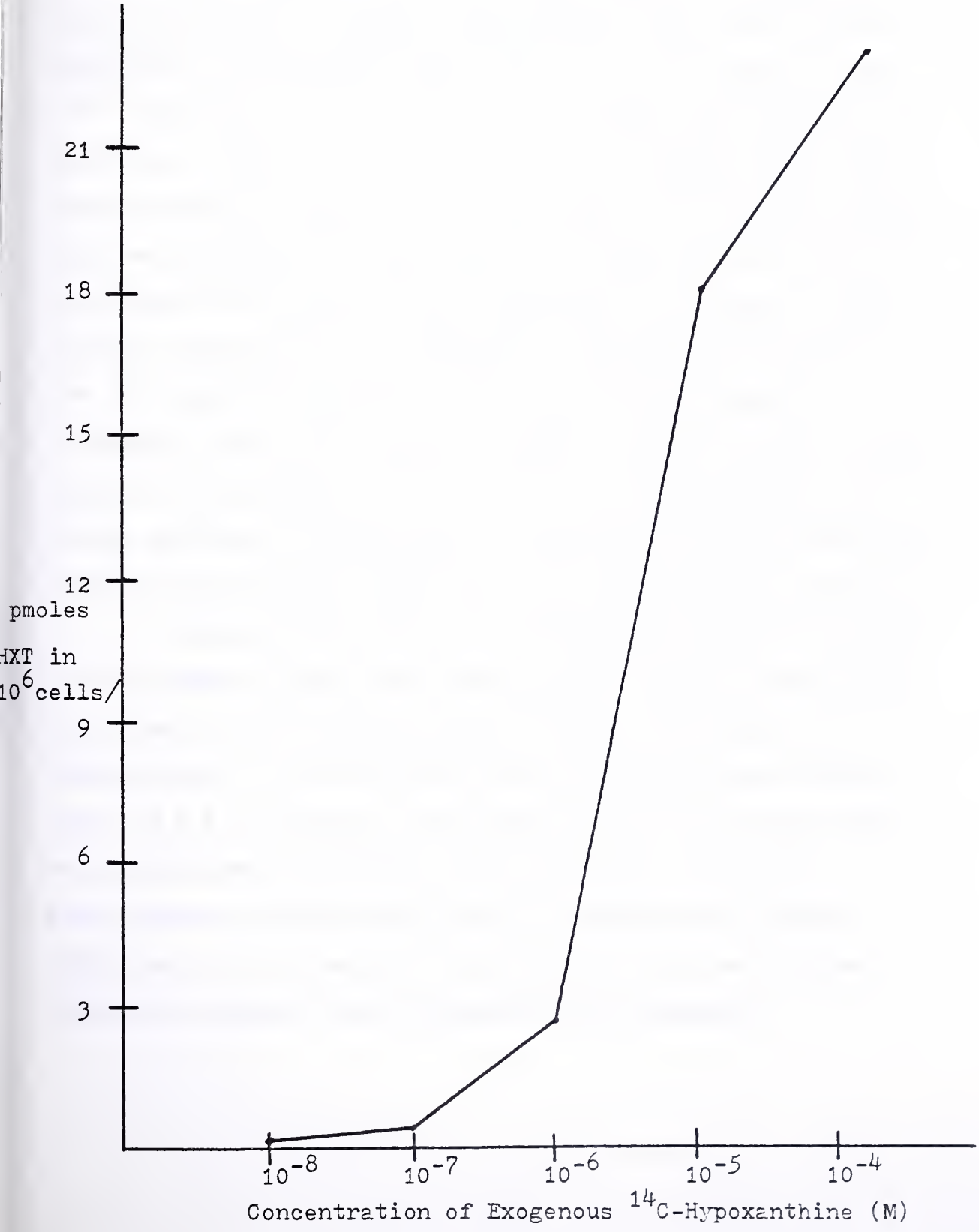
The effects of increasing concentrations of exogenous ^{14}C- hypoxanthine on salvage purine biosynthesis was tested to demonstrate the existence of the enzymatic machinery needed for salvage purine synthesis in L1210 cells. Figure E shows that a significant amount of ^{14}C-hypoxanthine was incorporated into adenine and guanine when the exogenous

Figure D Exogenous Hypoxanthine vs. the Rate of de novo Purine Synthesis



The control value for the rate of de novo purine synthesis with no exogenous hypoxanthine in the media was 10.2 pmoles ^{14}C-glycine into A+G/10^6 cells/hr.

Figure E Exogenous ^{14}C-Hypoxanthine vs. Rate of Salvage Purine Synthesis



concentration of ^{14}C-hypoxanthine was 1μM or greater. This experiment was performed on log phase cells at a density of 4.3 X 10^5cells/cc. Figure E demonstrates that when the concentration of exogenous hypoxanthine is in the range of 1μM, the L1210 cells will utilize this substrate in salvage purine synthesis. Figures D and E together demonstrate that de novo purine synthesis progressively decreases as the salvage purine synthesis pathway is increasingly utilized which it will be if a suitable substrate such as hypoxanthine is present in sufficient concentrations. Therefore, one possible explanation for the observed decrease in de novo purine synthesis throughout the logarithmic and plateau phases of L1210 culture growth is increasing utilization of the salvage pathway of purine synthesis occurring as cell death and purine catabolism provide the necessary substrates for salvage purine synthesis.

Intracellular pools of 1-^{14}C-glycine in early and middle log phase cultures were measured after cell cultures were incubated with 1-^{14}C-glycine for 24 hours. The amount of intracellular 1-^{14}C-glycine/10^6cells within cultures harvested at 9.0 X 10^4cells/cc and others at 3.8 X 10^5cells/cc was not significantly different after 24 hours. Therefore, growth phase specific variations in intracellular glycine pools could not account for the decrease in de novo purine synthesis observed with increasing culture density.

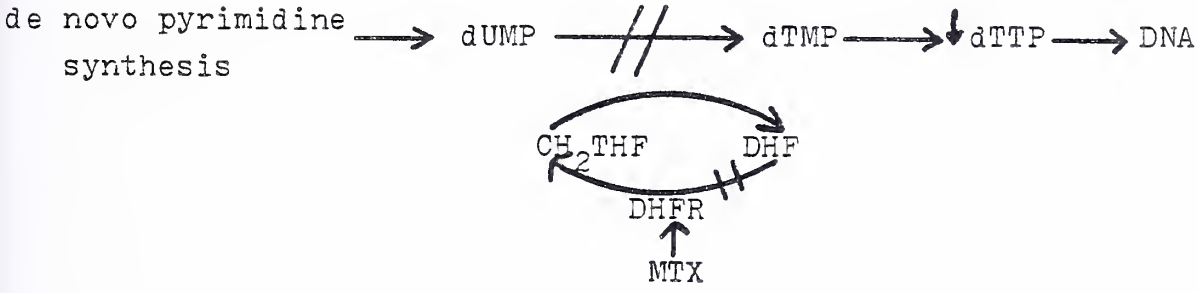
DISCUSSION

Effects of de novo Purine Synthesis Inhibitors on 5-Fluorouracil Metabolism and Cytotoxicity

In conjunction with the above work which quantitated the degree to which anti-tumor agents inhibited de novo purine synthesis, Cadman et al. examined the effects of five of these anti-purine agents on 5-fluorouracil metabolism and cytotoxicity. The results of the influence of methotrexate, tubercidin, 6-methylmercaptopurine ribonucleoside(MMPR), azaserine and L-alanosine on the intracellular accumulation of 5-fluorouracil and on PRPP levels are summarized in Table 4 (9).

As noted previously, the effect of the folate analog, methotrexate, in increasing the intracellular accumulation of 5-fluorouracil is thought to result from the inhibition of de novo purine synthesis by methotrexate. Methotrexate is a tight-binding inhibitor of dihydrofolate reductase. It was found to be a potent inhibitor of de novo purine synthesis, reducing it to 11.3% of control. Methotrexate prevents the conversion of dihydrofolate to 5,10-methylenetetrahydrofolate (CH_2THF), thereby depleting the CH_2THF pools.(See Figure F) CH_2THF is required for the conversion of deoxyuridylic acid (dUMP) to thymidylic acid(dTMP) and methotrexate therefore inhibits thymidylic acid synthesis in the de novo pyrimidine pathway. The CH_2THF pools are also necessary for methylation at two steps in de novo purine synthesis and therefore purine synthesis is significantly reduced with methotrexate.

Figure F Effect of Methotrexate on Thymidylate Synthesis



CH_2THF - 5,10 methylenetetrahydrofolate

DHF - dihydrofolate

DHFR - dihydrofolate reductase

MTX - Methotrexate

Table 4 Effects of Purine Synthesis Inhibitors on 5-Fluorouracil Accumulation and PRPP Levels (9)

Drug	Concentration (μM)	5-FU Accumulation (Treated/Control)	PRPP
Methotrexate	10	4.32	8
MMPR	10	4.40	15.7
Azaserine	10	3.00	15.0
L-Alanosine	10	1.80	25.0
Tubercidin	10	Undetected	0.1

In control cells, 5-fluorouracil accumulation was 0.025pmoles/min·10^6cells and PRPP was 7± 1.2ng/10^6cells. Cell cultures were exposed to the drugs for 3 hours before evaluating 5FU accumulation and PRPP levels.

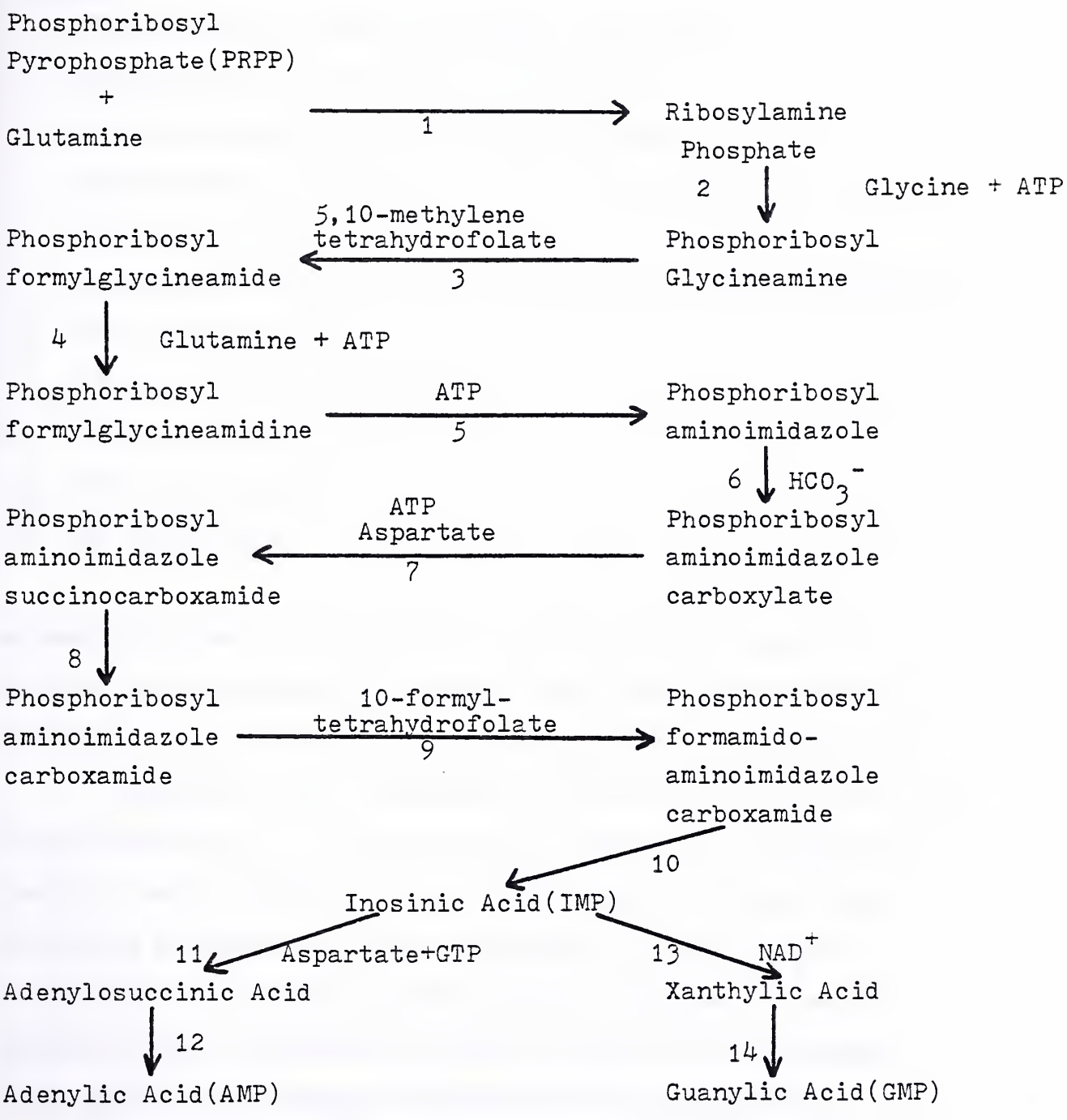
MMPR - 6-methyl-mercaptopurine ribonucleoside.

(See Figure G). This two point inhibition of de novo purine synthesis leads to a build-up of PRPP due to decreased utilization and to reduced feedback inhibition of the de novo pathway by purine nucleotide endproducts. Since PRPP synthetase is a regulatory enzyme inhibited by the purine endproducts(10), decreased purine synthesis results in increased PRPP synthesis and therefore in elevated intracellular PRPP levels. This PRPP is then available to enhance the conversion of 5-fluorouracil to 5-FUMP leading to increased intracellular accumulation of 5-fluorouracil. Hypoxanthine completely prevents the purineless state induced by methotrexate, a state which contributes to the lethal effects of methotrexate(11). However, both thymidine and a purine source are required by all cell lines examined to completely eliminate the cytotoxic effects of methotrexate(12).

Tubercidin(7-deazadenosine) is phosphorylated to its active metabolite by adenosine kinase. In its active form, it inhibits PRPP synthetase(13) and therefore does not result in increased PRPP levels nor in enhanced intracellular FUra accumulation. When L1210 cells, pretreated with methotrexate, were incubated with tubercidin, the PRPP elevation and increased FUra accumulation seen with methotrexate were prevented(3). This suggests that de novo purine synthesis inhibition resulting in increased PRPP levels is the key effect of methotrexate in augmenting FUra accumulation.

Another purine nucleoside analog, MMPR(6-methylmercaptopurine ribonucleoside), is also phosphorylated by

Figure G Pathway of de novo Purine Synthesis



ENZYMES

1. Amidophosphoribosyl tranferase
2. Phosphoribosylglycineamide synthetase
3. Phosphoribosylglycineamide formyltransferase

Figure G (continued)

ENZYMES

4. Phosphoribosylformylglycineamide synthetase
5. Phosphoribosylaminoimidazole synthetase
6. Phosphoribosylaminoimidazole carboxylase
7. Phosphoribosylaminoimidazole-succinocarboxamide synthetase
8. Adenylosuccinate lyase
9. Phosphoribosylaminoimidazole-carboxamide formyltransferase
10. IMP cyclohydrolase
11. Adenylosuccinate synthetase
12. Adenylosuccinate lyase
13. IMP dehydrogenase
14. GMP synthetase

adenosine kinase and inhibits the rate-limiting enzyme of de novo purine synthesis, amidophosphoribosyl transferase(14). MMPR did elevate PRPP levels and enhanced FUra accumulation.

Azaserine also increased intracellular PRPP levels and FUra accumulation. This anti-purine agent is a glutamine antagonist which inhibits de novo synthesis at the two steps requiring glutamine for amide transfer(15).(See Figure G) Inhibition of the first amide transfer catalyzed by amidophosphoribosyl transferase probably leads to the increased PRPP levels and subsequent enhanced FUra accumulation.

L-alanosine, an L-aspartate analog, competitively inhibits the intracellular transport of L-aspartic acid and inhibits several enzymes involved in de novo purine synthesis

including adenylosuccinate synthetase, phosphoribosylamino-imidazole-succinocarboxamide synthetase, and L-glutamine synthetase(9). Inhibiting de novo purine synthesis at the steps catalyzed by the above enzymes led to increased PRPP levels and to augmented FUra accumulation in the L1210 cultures treated with L-alanosine.

The inhibitory effects of these 5 agents on de novo purine synthesis is summarized from Table 1 in Table 5.

Table 5 Inhibition of de novo Purine Synthesis by Cytotoxic Agents

Drug	Exposure (hrs.)	Concentration (μM)	1-^{14}C-glycine into A+G/10^6cells/hr. (% of control $\pm$ 2.1%)
Methotrexate	3	10	11.3
MMPR	3	2	61.0
Azaserine	3	10	14.1
Tubercidin	1.5	10	17.4
L-alanosine	3	2	79.0

Each drug had a significant inhibitory effect on de novo purine synthesis and each agent, except L-alanosine, inhibited both adenine and guanine synthesis equally. L-alanosine inhibits adenylosuccinate synthetase which converts IMP to AMP. Thus, adenine synthesis is inhibited while the pathway from IMP to GMP is unaltered. The 21% reduction in purine synthesis seen with L-alanosine was due entirely to decreased adenine synthesis.

To examine potential cytotoxic synergism between these 5 anti-purine agents 5-fluorouracil, L1210 cultures were treated sequentially with an anti-purine drug and FUra and cytotoxicity was determined by soft agar cloning of the cells. The results are shown in Table 6 (9).

Table 6 Viability of L1210 Cells (9)

DRUG 1	DRUG 2	μM(1)	μM(2)	hr(1)	hr(2)	%Viability
Control	0	0	0	0	0	100
Methotrexate	0	10	0	4	0	60
0	FUra	0	10	0	1	100
Methotrexate	FUra	10	10	3 ⟶	1	10
MMPR	0	1	0	4	0	100
MMPR	FUra	1	10	3 ⟶	1	6
Azaserine	0	6	0	4	0	77
Azaserine	FUra	6	10	3 ⟶	1	10
L-alanosine	0	10	0	4	0	106
L-alanosine	FUra	10	3	3 ⟶	1	41

The arrow(⟶) indicates that Drug 1 was given for the indicated time before adding Drug 2. Total drug exposure was 4 hours before cloning the cells in soft agar.

Synergistic cytotoxicity was seen with the anti-purine agents which increased intracellular FUra accumulation. It therefore appears that pretreatment with anti-purine agents which results in increased PRPP levels, allows for increased activation of FUra to FUMP and for increased FUra accumulation, resulting, therefore, in enhanced cell killing.

This proposed mechanism of cytotoxic synergism is consistent with the findings of other investigators. MMPR increases intracellular PRPP levels and has been shown to enhance conversion of 6-thioguanine(6-TG) and 6-mercaptopurine (6-MP) to their active inhibitory metabolites, 6-thioguanylic acid and thioinosinic acid, respectively(16,17).

$$\text{6-MP} + \text{PRPP} \xrightarrow[\text{Phosphoribosyl transferase}]{\text{Guanine-Hypoxanthine}} \text{thio-IMP} + \text{PPi} \quad (18)$$

$$\text{6-TG} + \text{PRPP} \xrightarrow[\text{Phosphoribosyl transferase}]{\text{Guanine-Hypoxanthine}} \text{thio-GMP} + \text{PPi} \quad (19)$$

Cadman et al. also examined the intracellular metabolites of FUra in L1210 cells pretreated with methotrexate(3,20) and MMPR(unpublished results). FUMP, FUDP, FUTP and FdUMP were all elevated above control values in proportion to the increase in total intracellular FUra accumulation. The increase in these metabolites probably occurred as a consequence of increased PRPP levels since the primary metabolic pathway for FUra is conversion to FUMP by orotate phosphoribosyltransferase, using PRPP as a co-factor.

$$\text{FUra} + \text{PRPP} \xrightarrow[\text{phosphoribosyltransferase}]{\text{orotate}} \text{FUMP} + \text{PPi} \quad (4,21)$$

The enhanced cytotoxicity observed following sequential treatment with an anti-purine agent and FUra may be the result of increased intracellular FdUMP and FUTP levels. FdUMP inhibits thymidylate synthetase and FUTP is incorporated into RNA. Complete protection of mouse L cells from FUra cytotoxicity requires the presence of both thymidine and uridine in the media indicating that FdUMP and FUTP act together in

killing cells(22).

This study illustrates how one category of anti-tumor agents, the de novo purine synthesis inhibitors, can enhance the metabolic activation of another group of agents, in this case, the fluoropyrimidines. It also demonstrates one approach to designing biochemically rational sequential drug treatments in the search for synergistic tumor cytotoxicity.

Effect of Cytotoxic Agents on de novo Purine Synthesis

Ed Cadman et al. demonstrated that the anti-purine agents which enhanced the effects of FUra were those which led to increased intracellular PRPP levels. In addition to the 5 agents discussed above, several other anti-tumor drugs with various mechanisms of action were found to inhibit de novo purine synthesis.

Pyrimidines and Pyrimidine Analogs and Antagonists

Ara-C(1-B-D-Arabinofuranosylcytosine) is a deoxycytidine analog, the active metabolite of which is the triphosphate form, Ara-CTP. Ara-C exerts its cytotoxic effect through the inhibition of DNA polymerase, although incorporation of Ara-C into DNA may be an important contribution to its anti-tumor action(23). In the presence of Ara-C, de novo purine synthesis was found to be 85% of control. It is possible that inhibition of DNA polymerase with a concomitant decrease in DNA synthesis leads to a build-up of adenine and guanine nucleotides which then inhibit de novo purine synthesis. Increased intracellular concentrations of purine ribonucleotides have been found to inhibit the first committed step in de novo purine synthesis which is catalyzed by the enzyme amidophosphoribosyl transferase(24, 50). The increased purine

levels also inhibit phosphoribosyl pyrophosphate(PRPP) synthetase, the enzyme which supplies PRPP, an essential cofactor for amidophosphoribosyl transferase(10,51). Given this proposed mechanism for the inhibition of de novo purine synthesis by Ara-C, this agent would not be expected to enhance the cytotoxic effects of FUra since no significant increase in PRPP levels would occur with the elevated purine nucleotide levels inhibiting PRPP synthetase.

Thymidine(dThd) is a pyrimidine nucleoside which inhibits ribonucleotide reductase in its triphosphate form(dTTP). Inhibition of this enzyme is thought to result from feedback inhibition by expanded dTTP pools and has been found to cause reduced dCTP levels and therefore decreased DNA synthesis(25). With decreased dCTP levels limiting DNA synthesis, an initial increase in purine ribonucleotide pools due to decreased utilization may result. The observed reduction in de novo purine synthesis by 27.5% may have been the result of unused end-products of purine synthesis feeding back and inhibiting amidophosphoribosyl transferase and PRPP synthetase. Thymidine has been shown to increase incorporation of FUra into RNA and to increase the cytotoxic effects of FUra, though the mechanism for this has not been well-defined(26). This drug sequence has been found to enhance the interference with ribosomal RNA processing that occurs when FUra is used alone(27).

Pyrazofurin(4-hydroxy-5-B-D-ribofuranosylpyrazole-3-carboximide) is a nucleoside analog which when converted to its monophosphate form by adenosine kinase, inhibits orotidylate decarboxylase, the enzyme which converts orotidylate(OMP)

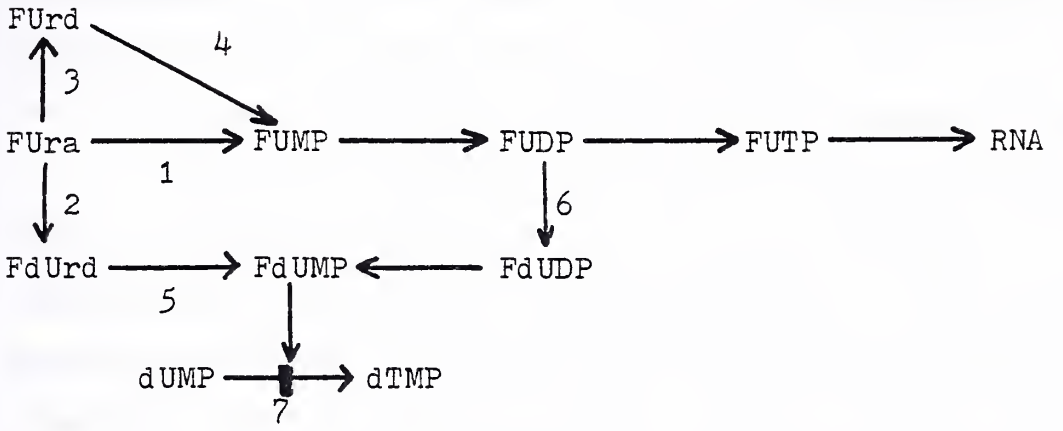
to uridylate(UMP) (28) in the de novo pyrimidine synthesis pathway. This results in reduced pyrimidine ribonucleotide and deoxyribonucleotide pools and in increased orotate levels as decreased UMP levels stimulate de novo pyrimidine synthesis(29). Pyrazofurin inhibited de novo purine synthesis only minimally, (93.8% of control) but this slight decrease may have been secondary to an increased utilization of PRPP in converting the elevated orotate pools to orotidylate. This effect of pyrazofurin in increasing consumption of PRPP by orotate phosphoribosyltransferase, will not only decrease de novo purine synthesis, but will antagonize the actions of FUra by decreasing FUra's conversion to FUMP, the first step in the metabolic activation of FUra. Studies of the enzyme kinetics have revealed that the conversion of orotate to OMP is energetically more favorable than the conversion of FUra to FUMP(4). Cadman et al. have examined the K_M values for the conversion of FUra and orotate to their 5'phosphate nucleotides by orotate phosphoribosyltransferase in L1210 cells and found them to be 520μM and 12μM, respectively(3). A.S. Bagnera, using Ehrlich ascites tumor cells, has shown that increased utilization of PRPP by phosphoribosyltransferases is a potent mechanism of de novo purine synthesis inhibition because the K_M for the phosphoribosyltransferases including orotate phosphoribosyltransferase, is in the range of 5-40μM, while the $K_M$for the amidophosphoribosyl transferase of de novo purine synthesis is 1mM(10).

5-azacytidine is a cytidine analog which is incorporated into RNA in the triphosphate form, 5-aza-CTP(31).

5-aza-CTP distorts the secondary structure of RNA and the ineffective, fragmented nucleic acid is rapidly degraded into its component nucleotides(32). Taylor et al. demonstrated increased total intracellular nucleotide pools in Chinese Hamster cells after a 2 hour treatment with 5-azacytidine. They also found a 50% reduction in PRPP levels presumed secondary to feedback inhibition of PRPP synthetase by the increased purine nucleotide pools(32). In this study 5-azacytidine was found to reduce de novo purine synthesis to 62.6% of control probably due to the degradation of faulty RNA molecules with the subsequent liberation of ATP and GTP. Pretreatment of cultures with 5-azacytidine would antagonize the cytotoxic effects of FUra because the decreased PRPP levels caused by 5-azacytidine would retard the conversion of FUra to its active metabolites.

5-fluorouracil(FUra) is a pyrimidine analog, the active metabolites of which are 5-FdUMP and FUTP.(See Figure H) 5-FdUMP inhibits thymidylate synthetase, the enzyme which converts dUMP to dTMP, thus, treatment with FUra results in dTTP deprivation. The "thymine-less death" is thought to occur as inadequate dTTP pools inhibit DNA synthesis leading to degradation of the fragmented pieces of DNA(33). FUTP is incorporated into RNA and there inhibits the processing and maturation of RNA precursor molecules which are subsequently degraded. FUTP also causes an inhibition of ribosome formation, miscoding during mRNA translation and mismatching of tRNA's and the appropriate amino acids(34,35). The reduction to 20.7% of control for de novo purine synthesis found after treating L1210 cells with FUra alone is probably the result of both the

Figure H Metabolic Conversion of 5-Fluorouracil (43)



Enzymes

1. Orotate phosphoribosyltransferase
2. Thymidine phosphorylase
3. Uridine phosphorylase
4. Uridine-cytidine kinase
5. Thymidine kinase
6. Ribonucleotide reductase
7. Thymidylate synthetase

"thymine-less" state and the production of faulty RNA molecules. Thymine deprivation leads to decreased DNA synthesis producing a build-up of purine nucleotides. The degradation of defective RNA molecules contributes to elevating the levels of purine nucleotides which inhibit de novo purine synthesis by feedback inhibition.

5-fluorouridine(FUrd), also a fluorinated pyrimidine, is a precursor of both the active metabolites of FUra, FdUMP and FUTP. (See Figure H) Therefore both degradation of faulty RNA molecules and inhibition of thymidylate synthetase with

the subsequent production of fragmented DNA molecules would result from treatment with FUrd. De novo purine synthesis in the presence of FUrd was only 26.1% of control probably because, as with FUra, the purine nucleotides liberated from the nucleic acid breakdown inhibited de novo purine synthesis.

5-fluoro-2'-deoxyuridine(FdUrd) is phosphorylated to FdUMP by thymidine kinase(36) and is cleaved to FUra by both thymidine and deoxyuridine phosphorylases(37). The primary metabolite of FdUrd is FdUMP which inhibits DNA synthesis by creating a thymine-deficient state. De novo purine synthesis was reduced to 53.3% of control with FdUrd probably as a result of the build-up of purine nucleotides following decreased DNA synthesis and from degradation of DNA fragments. Some conversion of FdUrd to FUra may also have contributed to FdUrd's anti-purine effect.

Purine Analogs and Antagonists

Hypoxanthine is a purine base which is converted to inosinic acid(IMP) by the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase. PRPP is the co-substrate in this conversion donating a phosphoribosyl moiety to hypoxanthine. IMP is then converted to AMP and GMP which feedback and inhibit de novo purine synthesis. Treatment of L1210 cultures with hypoxanthine decreased de novo purine synthesis to 13.0% of control by providing a substrate for the salvage pathway of purine biosynthesis. Because the salvage metabolism of hypoxanthine utilizes PRPP, hypoxanthine would be expected to antagonize the activation and therefore cytotoxic effects of

FUra. Cadman et al. found that adding hypoxanthine to L1210 cultures pretreated with methotrexate prevented the methotrexate-induced elevation of PRPP levels and enhanced intracellular accumulation of FUra(3).

Inosine is a purine nucleoside which inhibited de novo purine synthesis to 9.3% of control. Inosine can be hydrolyzed to hypoxanthine and the free ribose moiety by purine nucleosidase. Inosine may also be converted by purine nucleoside phosphorylase to hypoxanthine and ribose-1-phosphate.

$$\text{Inosine} \xrightarrow[\text{Pi}]{\text{Nucleoside Phosphorylase}} \text{Hypoxanthine} + \text{Ribose-1-Phosphate}$$

$$\text{Inosine} \xrightarrow[\text{Purine Nucleosidase}]{H_2O} \text{Hypoxanthine} + \text{Ribose} \qquad (38)$$

This hypoxanthine can then be converted to IMP by hypoxanthine-guanine phosphoribosyltransferase with PRPP acting as the phosphoribosyl donor. De novo purine synthesis is probably inhibited as the AMP and GMP formed from IMP feedback and inhibit amidophosphoribosyl transferase and PRPP synthetase. Utilization of PRPP in salvaging hypoxanthine may also contribute to the observed decrease in de novo purine synthesis(10). It has been shown that in cells such as L1210 cells which possess the enzyme purine nucleoside phosphorylase, inosine promotes the incorporation of FUra into RNA by providing a ribose-1-phosphate moiety which converts FUra to FUrd(39,40).

$$\text{FUra} + \text{Ribose-1-Phosphate} \xrightarrow[\text{Phosphorylase}]{\text{Uridine}} \text{5-Fluorouridine}$$

From FUrd, the activation of FUra proceeds to both FdUMP and

FUTP(See Figure H). Inosine, then, would be expected to enhance the cytotoxic effects of FUra by providing not PRPP, but ribose-1-phosphate to activate FUra.

Adenosine is a purine nucleoside which inhibited de novo purine synthesis to 20.7% of control. Adenosine can be converted to inosine by adenosine deaminase which, after metabolism to hypoxanthine, can be used in salvage purine biosynthesis. Adenosine can also be phosphorylated by adenosine kinase to AMP which will feedback and inhibit de novo purine synthesis. As with inosine, adenosine can be catabolized to its respective purine base, adenine, by nucleoside phosphorylase producing ribose-1-phosphate which can convert FUra to FUrd. Adenosine suppresses de novo purine synthesis by conversion to both AMP and hypoxanthine, the latter which is salvaged to IMP. Adenosine has been shown to markedly stimulate the uptake and incorporation of FUra into the RNA of Novikoff hepatoma cells by providing the ribose-1-phosphate for activation of FUra(41).

6-mercaptopurine(6-MP) is a hypoxanthine analog which inhibited de novo purine synthesis to 58.7% of control. 6-MP utilizes PRPP in its conversion to thio-IMP by hypoxanthine-guanine phosphoribosyltransferase(42). Thio-IMP is then converted to deoxy-thio-ITP and in this form may be incorporated into DNA. Thio-IMP prevents the conversion of IMP to AMP and GMP by inhibiting adenylosuccinate synthetase and IMP dehydrogenase. It also inhibits amidophosphoribosyl transferase by mimicking the effect of the natural purine nucleotides as negative feedback inhibitors(14). Thio-IMP's role as a pseudo-

feedback inhibitor is thought to be the major site of inhibition for 6-MP because the K_I fro thio-IMP in inhibiting amidophosphoribosyl transferase is 4.4×10^{-5}M, a concentration generally achieved in cell cultures and therapeutic regimens(42). 6-MP would be expected to compete with FUra for PRPP, the substrate both agents utilize for metabolic activation.

6-thioguanine(6-TG) is a guanine analog which inhibited de novo purine synthesis to 15.9% of control. Like 6-MP, 6-TG is converted to its monophosphate form, thio-GMP, by hypoxanthine-guanine phosphoribosyltransferase, utilizing PRPP as the phosphoribosyl donor. Thio-GMP acts as an allosteric pseudofeedback inhibitor of amidophosphoribosyl transferase, thus depressing de novo purine synthesis. Thio-GMP inhibits IMP dehydrogenase which converts inosinic acid(IMP) to xanthylic acid(XMP). Thio-GMP also inhibits the conversion of GMP to GDP by nucleotide monophosphokinase(42, 14), thus allowing the relative accumulation of GMP which will inhibit de novo purine synthesis by endproduct inhibition. 6-TG would be expected to antagonize the cytotoxic effects of FUra since both agents compete for metabolic activation by PRPP.

Virazole(1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a nucleoside analog which inhibits inosinate dehydrogenase, the enzyme which converts inosinic acid to xanthyic acid, the precursor of guanylic acid(GMP)(44). Virazole has been found to be structurally similar to guanosine in its crystalline state. Its active metabolite is virazole-5'-phosphate and it is thought to be a substrate for nucleoside kinase(44). Virazole decreased de novo purine synthesis

to 39.1% of control. Inhibition of guanine nucleotide synthesis was twice as great as inhibition of adenine synthesis. In addition to reducing the guanine pools directly, virazole may also have decreased AMP synthesis because GTP is a necessary cofactor in the conversion of IMP to adenylosuccinic acid(SAMP), the precursor of AMP. With both AMP and GMP synthesis decreased by virazole, the precursor IMP builds up and further decreases de novo purine synthesis by feedback inhibition of amidophosphoribosyl transferase(24) and PRPP synthetase(45). This inhibition of PRPP synthetase would result in decreased FUra activation making virazole and FUra an antagonistic sequential combination of agents.

Other Anti-Tumor Agents

PALA(N-phosphonacetyl-L-aspartate) is a transition state analog inhibitor of L-aspartate transcarbamoylase, the enzyme catalyzing the first committed step in de novo pyrimidine synthesis. Treatment with PALA leads to decreased pyrimidine ribonucleotide and deoxyribonucleotide pools and to a marked decrease in orotate synthesis(30). The reduced pyrimidine nucleotide levels limit DNA and RNA synthesis and purine nucleotides accumulate due to decreased utilization. PALA may interfere with de novo purine synthesis(85% of control) indirectly as the elevated purine levels reduce de novo purine synthesis by feedback inhibition. The reduced orotate levels would enhance the activation of FUra to FUMP because FUra competes with orotate which is the preferred substrate for orotate phosphoribosyltransferase(9).

Hydroxyurea inhibits ribonucleoside diphosphate reductase leading to reduced deoxynucleotide triphosphate pools and therefore to reduced DNA synthesis(46). Hydroxyurea produces increased levels of ADP and GDP which feedback and inhibit amidophosphoribosyl transferase(24,45), thus decreasing de novo purine synthesis. In this study hydroxyurea was found to decrease de novo purine synthesis only to 90% of control. Hydroxyurea would be expected to partially antagonize the effects of FUra by blocking the conversion of FUDP to FdUDP and therefore to the active metabolite, FdUMP, a reaction catalyzed by ribonucleotide diphosphate reductase. (See Figure H) FUDP, however, would still be converted to FUTP and incorporated into RNA in the presence of hydroxyurea. It has been reported that hydroxyurea does partially block the activation of FUra in Novikokk hepatoma cells(41).

Leukovorin(5-formyl-tetrahydrofolate) can be converted to 5,10 methylene tetrahydrofolate within cells and therefore can rescue L1210 cells from the anti-purine and anti-thymidine effects of methotrexate(47). As expected, leukovorin did not inhibit de novo purine synthesis, but rather, increased it slightly to 108.2% of control. Leukovorin would not be expected to be synergistic with FUra as no increase in intracellular PRPP would result from pretreatment with leukovorin.

Vincristine, a vinca alkaloid, binds with tubulin, the structural protein precursors of microtubules, preventing their assembly and inhibiting the mitotic spindle. Vincristine arrests mitosis in metaphase and the bound tubulin molecules

form intracellular, crystalline precipitates resulting in cell death and lysis(48). Following cell death, nucleic acid breakdown and purine salvage occur and the increased concentrations of liberated purines decrease de novo purine synthesis by feedback inhibition. Vincristine was found to inhibit de novo purine synthesis to 15.2% of control. No increase in intracellular PRPP would result from treatment with vincristine because the salvaged purines would inhibit PRPP synthetase as well as amidophosphoribosyl transferase. Therefore cytotoxic synergism resulting from sequential treatment with vincristine and FUra would be unlikely.

Actinomycin D is an antibiotic which non-covalently complexes with DNA preventing its template function in transcription. Actinomycin D's phenoxazone ring intercalates between two G-C base pairs blocking RNA chain elongation by RNA polymerase(49). The non-functional DNA-actinomycin D complexes are rapidly degraded producing increased intracellular ATP and GTP levels from DNA and RNA breakdown. The elevated nucleotide levels feedback and inhibit de novo purine synthesis regulating both amidopohsphoribosyl transferase and PRPP synthetase. In one study, a 50% reduction in intracellular PRPP levels was found after incubating Chinese Hamster cells with actinomycin D for 2 hours, suggesting feedback inhibition by a measured two-fold increase in intracellular purine nucleotides(32). In this study actinomycin D was found to be a potent inhibitor of de novo purine synthesis, reducing the rate to 8.7% of control. With the lowered PRPP levels that actinomycin D induces, less FUra would be converted to the

metabolically active fluoro-nucleotides.

Cycloheximide is a protein synthesis inhibitor which binds to ribosomal RNA inhibiting the peptidyl transferase activity of the 60S ribosomal subunit(50). These faulty RNA molecules are rapidly degraded and the liberated purines are salvaged. As with actinomycin D, de novo purine synthesis is inhibited by these nucleotides made available by nucleic acid catabolism. Cycloheximide decreased de novo purine synthesis to 10.9% of control in this study. Taylor et al. found a 50% reduction in PRPP levels following a 2 hour incubation with cycloheximide and a concomitant two-fold increase in purine nucleotides(32). Pretreatment of cultures with cycloheximide would antagonize the effects of FUra because the decreased PRPP levels would decrease activation of FUra.

De Novo Purine Synthesis in relation to Cell Culture Density

Studies examining the rate of de novo purine synthesis along the growth curve of L1210 cells revealed that the rate of de novo purine synthesis decreased steadily with increasing cell culture density, even during the exponential phase of growth. Possible causes for this decline in de novo purine synthesis in the face of logarithmic cell growth include: 1) increased salvage purine synthesis secondary to either decreased availability of 1-^{14}C-glycine in older, more populated cultures or to increased salvage pathway substrate availability, 2) a decreased rate of de novo purine synthesis secondary to a lengthening of the cell cycle along the growth curve, and 3) decreasing cell volume with less purine and

pyrimidine synthesis required to maintain stable intracellular nucleotide concentrations. The observed decrease in de novo purine synthesis along the growth curve represents a rate measurement because cell cultures at varying densities were each incubated with 1-^{14}C-glycine for 2 hours before the amount of newly synthesized adenine and guanine was quantitated. Because the cell cultures were growing logarithmically during this study, the total amount of purine synthesis was necessarily increasing as the cell culture density increased along the growth curve.

One explanantion for the decrease in de novo purine synthesis in the face of logarithmic culture growth is that salvage purine biosynthesis is supplying increasing amounts of purine nucleotides with increasing culture density. It was demonstrated above that the amount of intracellular 1-^{14}C-glycine per 10^6cells in old and new cultures with densities of 3.8 X 10^5cells/cc and 9.0 X 10^4cells/cc, respectively, after a 24-hour incubation with 1-^{14}C-glycine was not significantly different. Therefore, decreasing availability of this radioactive substrate at higher culture densities could not account for the observed decline in de novo purine synthesis.

Increasing concentrations of exogenous hypoxanthine in the culture media led to a steady decrease in de novo purine synthesis, and with increasing concentrations of exogenous ^{14}C-hypoxanthine, proportionately greater amounts of adenine and guanine were synthesized from ^{14}C-hypoxanthine. These results reveal that L1210 cells possess hypoxanthine-guanine phosphoribosyltransferase, the purine salvage enzyme, and that

hypoxanthine is the preferred substrate for purine synthesis. Given the choice, L1210 cell cultures will utilize the salvage rather than the de novo pathway for purine biosynthesis. The K_M of rat liver hypoxanthine-guanine phosphoribosyltransferase for PRPP is 5uM(52) and of adenine phosphoribosyltransferase is 6uM(53). This contrasts with K_M values of mammalian amidophosphoribosyl transferase for PRPP of 86-600uM(54). Thus the salvage pathway may inhibit de novo synthesis not only by endproduct inhibition of amidophosphoribosyl transferase and PRPP synthetase, but also by preferential utilization of PRPP.

In order for the purine salvage pathway to become increasingly active during logarithmic growth, cell death with reutilization of nucleic acids must also increase proportionately during log growth. The viability of L1210 suspension cultures as tested by trypan blue exclusion was found to be 99% during exponential growth. After 120 hours of growth in culture, viability was reduced to $\leq$ 70%(7). It has been suggested that since cell lysis rapidly follows cell death, the trypan blue exclusion test may not accurately measure the total amount of cell death(55). But even if the percentage of dying cells was slightly greater than 1% during log growth, it is unlikely that enough cell death and subsequent purine salvage could occur during the log phase to compensate for the observed decrease in de novo purine synthesis to 20% of control. In a suspension culture of human lymphoid cells, the percentage of cells in S phase, actively synthesizing DNA, was 50% during exponential growth(55). Thus much more than 1% of the cells would have to undergo lysis along the

growth curve to allow the salvage pathway to significantly contribute to purine synthesis during log growth. During plateau phase growth, however, in a cell culture that is not nutritionally depleted, the percentage of cells in S phase is equal to the percentage of dying cells, thus maintaining the plateau phase steady state(55). In this situation, the salvage pathway of purine synthesis would be expected to substantially contribute to total purine biosynthesis.

A second possible explanation for the reduced rate of de novo purine synthesis during logarithmic growth is elongation of the cell cycle resulting in a slower overall rate of de novo synthesis, not necessarily accompanied by a decrease in total de novo purine synthesis. The cell cycle is defined as the interval between the midpoint of mitosis and the midpoint of the subsequent mitosis of one or both daughter cells (56). It has been found that the control of population growth in a cell culture may be due to nutritional deficiency or to cell-cell interactions and density inhibition(55,56). Holly and Kiernan demonstrated that the concentration of serum in culture media determined the final saturation density of cultured cells(57). Yen et al., however, have shown that for human lymphoid cells in suspension cultures at a concentration of 6.5-7.0 X 10^6cells/cc, no increment in serum concentration would stimulate continued culture growth. This shows that at 7 X 10^6cells/cc, culture density becomes the growth limiting mechanism for human lymphoid cells(55). L1210 cultures passing through the exponential and plateau phases of growth in the inoculate's original media, are likely growth regulated by both

inhibitory mechanisms. The cells of cultures in which nutritional deficiencies develop over time progressively accumulate in the G_1 phase of the cell cycle(55,58), the interval between completion of mitosis and the onset of DNA synthesis(56). Rossow et al. have proposed that during G_1, an intracellular protein (R) is synthesized and when accumulated in sufficient quantities, allows for the initiation of DNA synthesis and for the completion of the cell cycle(58). Serum growth factors are believed to induce this "initiator protein". Thus in nutritionally depleted cultures, cells build up in an elongated G_1 phase presumably because more time is required for the initiator protein concentration to reach the critical levels. During logarithmic growth, the nutritive serum factors are gradually consumed resulting in elongated G_1 phases and thus in a lengthening of the cell cycle along the growth curve. As increasing numbers of cells accumulate in the G_1 phase, a decreasing percentage of cells will be synthesizing DNA (S phase) as logarithmic growth progresses. This gradual decrease in the percentage of S phase cells along the growth curve has been demonstrated by many investigators(11,59). In L1210 suspension cultures, thymidine labelling indices decline nearly 10-fold throughout exponential and plateau phases of growth(11). This decreasing percentage of S phase cells coupled with a lengthening cell cycle offers an explanation for the observed decreased rate of de novo purine synthesis in the face of exponential cell culture growth. Per unit time, fewer and fewer cells will be actively involved in de novo purine synthesis as logarithmic growth progresses, although

the total amount of de novo purine synthesis will increase as culture doublings continue along the growth curve. Hahn et al. observed a gradual lengthening of the cell cycle in Chinese Hamster cells with 15 hours being the average cell cycle for early log phase cells and 32 hours the average cycling time for plateau phase cells(60). The growth curve for L1210 cells in Figure B demonstrates this elongation of the cell cycle with 10, 22 and $>$ 44 hours being required for successive cell culture doublings during exponential and plateau phase growth.

A third phenomenon possibly contributing to the explanation for the decreased rate of de novo purine synthesis during logarithmic growth is a progressive decrease in cell volume along the growth curve thereby requiring fewer nucleotides per cell to maintain constant nucleotide concentrations. If cell volume gradually decreased, less total purine synthesis would have to occur to supply a growing culture of smaller cells with the purines needed to achieve the appropriate nucleotide concentrations. Yen et al. demonstrated that the cells in nutritionally limited cultures gradually accumulate in G_1 and that their volumes progressively diminish(55). C. Benz et al. showed that in L1210 suspension cultures, cell volumes decrease throughout the mid log, late log and plateau phases of growth, being reduced to 60% of their initial volumes by the late log and plateau phases(7). Scanning electron microscopy of these smaller cells revealed them to be of G_0/G_1 appearance with loss of surface microvilli(61). Benz et al. also demonstrated a progressive decrease in intracellular deoxynucleotide triphosphate pools/10^6 cells with increasing culture density, reflecting the need for reduced nucleotide pools with decreasing cell volume(7).

De novo purine synthesis will decraese in response to the need for reduced nucleotide pools which develop as cell volumes decrease during log growth.

In examining possible causes for the decreased rate of de novo purine synthesis throughout log and plateau phase growth, it seems likely that increased salvage pathway purine synthesis, elongation of the cell cycle resulting in fewer S phase cells per unit time, and decreased cell volume along the growth curve all contribute to the reduced rate of de novo synthesis. Given that the rate of de novo purine synthesis in late log phase has decreased to $\sim$ 20% of its early log phase value, the anti-purine effects of an anti-tumor agent must be interpreted keeping this natural decrease in mind. As seen in Table 2, methotrexate's anti-purine effects could be underestimated if tested only with late log phase cells; methotrexate is a very potent anti-purine agent when incubated with early log cells which are synthesizing larger quantities of purines per unit time. Conversely, the anti-purine effects of a weakly inhibitory anti-tumor agent could be overrated if tested with late log phase cells which have decreased rates of de novo purine synthesis for reasons unrelated to the cytotoxic agent. The anti-purine effects of drugs, therefore, must be studied utilizing control cultures with nutritional states and culture densities identical to those of the experimental cultures.

5-fluorouracil exhibits cytotoxic synergism with anti-purine agents which elevate intracellular levels of PRPP resulting in enhanced metabolic activation of the fluoropyrimidine. The synergism between, for example, methotrexate and

FUra, however, is related to the anti-purine potency of methotrexate because the degree of PRPP level elevation is proportional to the magnitude of methotrexate's inhibition of de novo purine synthesis(9). Because intracellular PRPP levels also decrease during log growth paralleling the rate of de novo purine synthesis, the synergism between methotrexate and FUra would be blunted if methotrexate were incubated with a late log culture in which the rate of de novo purine synthesis was only 20% of the maximal early log rate.(7) Similarly, the synergistic effects of sequential treatment with methotrexate and FUra seen with early log phase cultures <u>in vitro</u> would probably not be as apparent when tested against a solid tumor mass <u>in vivo</u>, the center of which contains cells in the G_0/G_1 resting phase due to hypoxia and nutrient depletion(62). The cell cycle kinetics of these resting tumor cells are more comparable to those of plateau phase cells in a nutrient- depleted culture and it is likely that the rate of de novo purine synthesis within this cell population would not be substantial enough to allow for marked inhibition by methotrexate. With methotrexate powerless to significantly inhibit low baseline levels of de novo purine synthesis in slowly cycling tumor cells, the already low baseline levels of PRPP would be elevated only slightly and the basis for the synergism between methotrexate and FUra would be undermined.

On the other hand, several investigations have revealed that some malignant tumors have an increased capacity for de novo purine synthesis. Measurments of amidophosphoribosyl transferase in rat hepatomas demonstrated a 2 to 4-fold

increase in activity compared to normal rat liver(63). This increased enzyme activity was found even in well-differentiated hepatomas with slow growth rates. Increased amidophosphoribosyl transferase activity has also been observed in transplantable rat kidney tumors relative to normal kidney cortex(64). AICAR (aminoimidazolecarboxamide ribonucleotide) transformylase and adenylosuccinate lyase, two other enzymes of the de novo purine synthesis pathway, have also been shown to have increased activity in rat hepatomas as compared to controls(65,66). Some malignant tumors, therefore, appear to have an increased capacity for de novo purine biosynthesis and, like early log phase cells in vitro, would likely be strongly inhibited by anti-purine agents and by sequential methotrexate-5-fluorouracil chemotherapy.

In summary, it has been shown that many anti-tumor agents with widely varying mechanisms of action have the ability to inhibit de novo purine synthesis. Even agents which do not directly interfere with the de novo purine synthesis pathway can profoundly affect purine biosynthesis. It is hoped that this knowledge of the anti-purine effects of the cytotoxic agents will be useful to other investigators involved in designing biochemically rational combination chemotherapy.

REFERENCES

1. Sartorelli, A.C. Approaches to the combination chemotherapy of transplantable neoplasms. Progr. Exp. Tumor Res. 6: 228-288, 1965.

2. Chabner, B. and Johns, D. Folate Antagonists. In Cancer - A Comprehensive Treatise, (F.F. Becker, ed.), Plenum Press, N.Y., Vol. 5, pp. 363-377, 1977.

3. Cadman, E., Benz, C. and Heimer, R. The influence of methotrexate pretreatment on 5-fluorouracil metabolism in L1210 cells. J. Biol. Chem.,256: 1695-1704, 1981.

4. Reyes, P. The synthesis of 5-fluorouridine-5-phosphate by a pyrimidine phosphoribosyltransferase of mammalian origin. 1. Some prpoerties of the enzyme from P-1534J mouse leukemia cells. Biochemistry, 8: 2057-2062, 1969.

5. Benz, C., Schoenberg, M., Choti, M. and Cadman, E. Schedule-dependent cytotoxicity of methotrexate and 5-fluorouracil in human colon and breast tumor cell lines. J. Clin. Invest., 66: 1162-1165, 1980.

6. Benz, C. and Cadman, E. Modulation of 5-fluorouracil metabolism and cytotoxicity by antimetabolite pretreatment in human colorectal adenocarcinoma HCT-8. Cancer Research, 41: 994-999, 1981.

7. Benz, C. and Cadman, E. Biochemical alterations during unperturbed suspension growth of L1210 cells. Cancer Res., 41: 157-163, 1981.

8. Hryniuk, W.M., Brox, L.W., Henderson, J.F. and Tamaoki, T. Consequences of methotrexate inhibition of purine biosynthesis in L5178Y cells. Cancer Res., 35: 1427-1432, 1975.

9. Cadman,E., Benz, C., Heimer, R. and O'Shaughnessy, J. Effect of de novo purine synthesis inhibitors on 5-fluorouracil metabolism and cytotoxicity. Biochemical Pharmacology, 30:2469-2472, 1981.

10. Bagnara, A.S., Letter, A.A. and Henderson, J.F. Multiple mechanisms of regulation of purine biosynthesis de novo in intact tumor cells. Biochimica et Biophysica Acta, 374: 259-270,1974.

11. Hryniuk, W.M. Purineless death as a link between growth rate and cytotoxicity by methotrexate. Cancer Res.,32: 1506-1511, 1972.

12. Moran, R.G., Mulkins, M. and Heidelberger, C. Role of thymidylate synthetase activity in the development of methotrexate cytotoxicity. Proc. Nat'l Acad. Sci., 76: 5924-5928, 1979.

13. Henderson, J.F. and Khoo, M. On the mechanism of feedback inhibition of purine biosynthesis de novo in Ehrlich ascites tumor cells in vitro. J. Biol. Chem., 240:3104-3109,1965.

14. Paterson, A.R.P. and Tidd,D.M. 6-thiopurines in Antineoplastic and Immunosuppressive Agents. Part II,(A.C. Sartorelli and D.G. Johns, eds.), Springer, N.Y., pp. 385-403, 1975.

15. Bennett, L.L.,Jr. Glutamine antagonists in Antineoplastic and Immunosuppressive Agents. Part II,(A.C. Sartorelli and D.G. Johns, eds.), Springer, N.Y., pp. 434-445,1975.

16. Nelson, J.A. and Parks, R.E., Jr. Biochemical mechanisms for the synergism between 6-thioguanine and 6-methylmercaptopurine ribonucleoside in Sarcoma 180 cells. Cancer

Res., 32:2034-2041,1972.

17. Scholar, E.M., Brown, P.R. and Parks. R.E.,Jr. Synergistic effect of 6-mercaptopurine and 6-methylmercaptopurine ribonucleoside on the levels of adenine nucleotides of Sarcoma 180 cells. Cancer Res., 32:259-269, 1972.
18. Brockman, R.W. and Anderson, E.P. Biochemistry of cancer (metabolic aspects). Ann. Rev. Biochem., 32:463-475,1963.
19. Brockman, R.W. Mechanisms of resistance to anticancer agents. Advances in Cancer Res., 7: 129-140,1963.
20. Cadman, E., Heimer, R. and Davis, L. Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: Explanation for drug synergism. Science,205: 1135-1137,1979.
21. Laskin, J.D., Evans, R.M., Slocum, H.K., Burke, D. and Hakala, M.T. Basis for natural variation in sensitivity to 5-fluorouracil in mouse and human cells in culture. Cancer Res., 39:383-390,1979.
22. Madoc-Jones, H. and Bruce, W.R. On the mechanism of the lethal action of 5-fluorouracil on mouse L cells. Cancer Res., 28:1976-1981,1968.
23. Kufe, D.W., Major, P.O., Egan, E.M. and Beardsley, G.P. Correlation of cytotoxicity with incorporation of Ara-C into DNA. T. of Biol. Chem., 255:8997-9000,1980.
24. Henderson, J.F. and Patterson, A.R.P. Nucleotide Metabolism, an Introduction. Academic Press, N.Y., p. 117,1973.
25. Howell, S.B. Thymidine as a chemotherapeutic agent: Sensitivity of human solid tumor and marrow progenitor cells in vitro. In Nucleosides and Cancer Treatment,(R.M.N. Tattersal and R.M. Fox, eds.), Academic Press, Austrailia,

pp.229-231, 1981.

26. Spiegelman, S., Sawyer, R., Nayak, R., Ritzi, E., Stolfi, R., and Martin, D. Improving the anti-tumor activity of 5-fluorouracil by increasing its incorporation into RNA via metabolic modulation. Proc. Nat'l Acad. Sci., 77: 4966-4970, 1980.

27. Carrico, C.K., and Glazer, R.I. Augmentation by thymidine of the incorporation and distribution of 5-fluorouracil in ribosomal RNA. Biochemical and Biophysical Research Communications, 87:664-670, 1979.

28. Cadman, E. and Benz, C. Uridine and Cytidine metabolism following inhibition of de novo pyrimidine synthesis by pyrazofurin. Biochimica and Biophysica Acta, 609:372-382, 1980.

29. Cadman, E.C., Dix, D.E. and Handschumacher, R.E. Clinical, biological, and biochemical effects of pyrazofurin. Cancer Res., 38: 682-688, 1978.

30. Moyer, J.D. and Handschumacher, R.F. Selective inhibition of pyrimidine synthesis and depletion of nucleotide pools by N-(phosphonacetyl)-L-aspartate. Cancer Res., 39: 3089-3094, 1979.

31. Vesely, J. and Cihak, A. 5-azacytidine: Mechanism of action and biological effects in mammalian cells. Pharmac. Ther. A. Vol. 2: 813-840, 1978.

32. Taylor, M.W., Olivelle, S., Levine, R.A., Coy, K., Hershey, H., Gupta, K.C. and Zawistowich, L. Regulation of de novo purine biosynthesis in Chinese Hamster cells. J. of Biol. Chem., 257:377-380, 1982.

33. Cohen, S.S. On the nature of thymine-less death. Annals of

the New York Academy of Sciences, 186:292-301, 1972.

34. Sawyer, R.C. and Stolfi, R.L. Mechanism of cytotoxicity in 5-fluorouracil chemotherapy of two murine solid tumors. In Nucleosides and Cancer Treatment, (H.M.N. Tattersal and R.M. Fox, eds.), Academic Press, Austrailia, pp.309-335, 1981.

35. Heidelberger, C. Fluorinated pyrimidines and their nucleosides. In Antineoplastic and Immunosuppressive Agents. Part II., (A.C. Sartorelli and D.G. Johns, eds.), Springer, N.Y., pp.193-231, 1975.

36. Harbers, E., Chauduri, N.K. and Heidelberger, C. Studies on fluorinated pyrimidines. VIII. Further biochemical and metabolic investigations. J. of Biol. Chem., 234:1255-1262, 1959.

37. Birnie, G.D., Kroeger, H. and Heidelberger, C. Studies on fluroinated pyrimidines. XVIII. The degradation of 5-fluoro-2'-deoxyuridine and related compounds by nucleoside phosphorylase. Biochemistry, 2:566-572, 1963.

38. Lehninger, A.L. Biochemistry, Worth Publishers, Inc., New York, pp.741-743, 1975.

39. Gotto,A.M., Belkhode, M.L. and Touster, O. Stimulatory effects of inosine and deoxyinosine on the incorporation of uracil-2-^{14}C, 5-fluorouracil-2-^{14}C, and 5-bromouracil-2-^{14}C into nucleic acids by Ehrlich ascites tumor cells in vitro. Cancer Res., 29: 807-811, 1969.

40. Kessel, D. and Hall, T.C. Influence of ribose donors on the action of 5-fluorouracil. Cancer Res., 29:1749-1754, 1969.

41. Wilkinson, D.S. and Crumley, J. Metabolism of 5-fluorouracil in sensitive and resistant Novikoff hepatoma cells.

J. of Biol. Chem., 252:1051-1056, 1977.

42. Roy-Burman, P. Recent Results in Cancer Research; Analogues of Nucleic Acid Components, Springer-Verlag, New York, pp. 16-27, 1970.

43. Cadman, E., Grant, S. and Benz, C. Biochemical Modulation as a guide to rational combination chemotherapy for the treatment of cancer. In Pediatric Oncology II, Martinus Nijhoff, Hingham, in press.

44. Streeter, D.G., Watkowski, J.T., Khare, G.P., Sidwell, R.W., Bauer, R.J., Robins, R.K. and Simon, L.N. Mechanism of action of 1-B-D- ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad spectrum antiviral agent. Proc. Nat'l Acad. Sci., 70:1174-1178, 1973.

45. Stryer, L. Bichemistry, W.H. Freeman and Co., San Francisco, p.537, 1975.

46. Krakoff, J.H., Brown, N.C. and Reichard, P. Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Cancer Res., 28:1559-1565, 1968.

47. Hakala, M.T. and Taylor, E. The ability of purine and thymidine derivatives and of glycine to support the growth of mammalian cells in culture. J.of Biol. Chem., 234: pp.126-128, 1959.

48. Goodman, L.S. and Gilman, A., eds. The Pharmacological Basis of Therapeutics, MacMillan Publishing Co., Inc., New York, pp.1284-1287, 1975.

49. Sobell, H.M. How actinomycin D binds to DNA. Scientific American, August, 1974, pp.82-91.

50. Rowe, P.B., McCairns, E., Madsen, G., Sauer, D. and Elliott,H.

De novo purine synthesis in avian liver. J. of Biol. Chem., 253:7711-7721, 1978.

51. Fox, I.H. and Kelley, W.N. Human phosphoribosylpyrophosphate synthetase. J. of Biol. Chem., 247:2126-2131, 1972.

52. Wohlheuter, R.M. Hypoxanthine phosphoribosyltransferase activity in normal, developing and neoplastic tissues of the rat. Eur. J. Cancer, 11:463-472, 1975.

53. Atkinson, M.R. and Murray, A.W. Inhibition of purine phosphoribosyltransferases of Ehrlich ascites tumor cells by 6-mercaptopurine. Biochem. J., 94:64-70, 1965.

54. Tsuda, M., Katunuma, N, Morris, H.P. and Weber, G. Purification, properties and immunotitration of hepatoma glutamine phosphoribosylpyrophosphate amidotransferase(amidophosphoribosyl transferase, EC 2.4.2.14). Cancer Res.,39: 305-311, 1979.

55. Yen, A., Fried, J. and Clarkson, B. Alternative Modes of population growth inhibition in a human lymphoid cell line growing in suspension. Experimantal Cell Research, 107: 325-341, 1977.

56. Baserga, R. Multiplication and Division in Mammalian Cells, Marcel Dekker, Inc., New York, p.163,1976.

57. Holley, R.W. and Kiernan, J.A. "Contact inhibition" of cell division in 3T3 cells. Proc. Nat'l Acad. Sci.,60: 300-304, 1968.

58. Rossow, P.W., Riddle, V.G.H. and Pardee, A.B. Synthesis of labile, serum-dependent protein in early G_1 controls animal cell growth. In Readings in Mammalian Cell Culture, 2nd edition, (R. Pollack, ed.), Cold Spring Harbor Labora-

tory, pp.421-425,1981.

59. Nordenskjold, B.A., Skoog, L., Brown, N.C. and Reichard, P. Deoxyribonucleotide pools and ribonucleotide pools in cultured mouse embryo cells. J. Biol. Chem.,245:5360-5368, 1970.

60. Hahn, G.M., Stewart, J.R., Yang, S.J. and Parker, V. Chinese Hamster cell monolayer cultures. Exp. Cell Research, 49: 285-292, 1968.

61. Knutton, S., Sumner, M.C.B. and Pasternak, C.A. Role of microvilli in surface changes of synchronized P815Y mastocytoma cells. J. Cell Biol., 66:568-576, 1975.

62. Valeriote, F. and Van Putten, L. Proliferation dependent cytotoxicity of anticancer agents:A review. Cancer Res., 35:2619-2630, 1975.

63. Katunuma, N. and Weber, G. Glutamine Phosphoribosylpyrophosphate amidotransferase: Increased activity in hepatomas. FEBS Letters, 49:53-56, 1974.

64. Prajda, N., Morris, H.P. and Weber, G.Glutamine phosphoribosylpyrophosphate amidotransferase(amidophosphoribosyl transferase, EC 2.4.2.14) activity in normal, differentiating and neoplastic kidney. Cancer Res.,39:3909-3914, 1979.

65. Worzalla, J.F., Sweeney, M.J. and Hodes, M.E. Phosphoribosylaminoimidazolecarboxamide formyltransferase activities in Morris hepatomas. Proc. Amer. Assoc. Cancer Res.,17: p.181, 1976.

66. Jackson, R.C., Morris, H.P. and Weber, G. Increased adenylosuccinase activity in hepatomas and kidney tumors. Life Sciences,18:1043-1048,1976.

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